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FAILURE OF IMMUNE SERA TO NEUTRALIZE DENGUE-2 VIRUS IN INTRATHORACICALLY INOCULATED *AEDES AEGYPTI*

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ABSTRACT. *Aedes aegypti* became infected when inoculated with a mixture of dengue-2 virus and anti-dengue-2 antibodies, but not when they were exposed to the same mixture *per os.* This phenomenon merits more detailed investigation. Understanding why this difference in mosquito infection rate occurs may lead to improved dengue virus assays or provide insights into the nature of dengue virus–antibody interactions.

Citing unpublished data, Rosen et al. (1989) reported that mixtures of dengue virus and anti-dengue antibodies readily infected *Aedes albopictus* (Skuse) and *Toxorhynchites amboinensis* (Doleschall) when inoculated into the mosquito's thorax. They speculated that the virus–antibody complexes became dissociated in the mosquito's hemocoel, rendering the virus infectious.

Herein, we report data that support and expand the observation of Rosen et al. (1989). Our study was not originally designed to investigate differences in mosquito infection rates associated with different routes of infection. Rather, we were attempting to quantify the ability of immune serum to neutralize dengue virus when a mixture of virus and serum is imbibed by *Aedes aegypti* (Linn.) (Fig. 1).

For our experiments we used an F_2 generation of *Ae. aegypti* from San Juan, Puerto Rico. Larvae were reared in an environmental chamber at 26°C and at low densities (200 larvae/28.5 × 24 × 4.5-cm plastic rearing tray). Adults exposed to virus were held at 30°C, 80% RH, and a 12:12 (L:D) photoperiod for 10 days of extrinsic incubation.

The Rexville strain of *Ae. aegypti,* a colony that was initiated from larvae collected in Puerto Rico during 1981 (Scott et al. 1993), was used for amplifying dengue-2 viruses and as recipient mosquitoes when we titered virus–blood suspensions by mosquito inoculation. These mosquitoes were reared as described above, but at higher larval densities (400–500 larvae/tray).

We used a dengue-2 virus strain isolated in 1986 from a 5-month-old infant who became ill and died in San Juan, Puerto Rico. Prior to our experiment, this isolate underwent 2 passages in *Tx. amboinensis.* It had a titer of $10^{6.6}$ 50% mosquito infectious doses (MID_{50}) per 1.0 ml.

To make the virus–blood suspension, we first inoculated *Ae. aegypti* (Rexville strain) with 76 MID_{50} of dengue-2 virus. After a 10-day incubation at 30°C, inoculated mosquitoes were used to make a virus suspension by grinding 20–25 mosquitoes in 0.5 ml of either heat-inactivated fetal calf serum or human immune sera. The virus–blood suspension was made by mixing 10 parts of the virus suspension with 9 parts triple-washed human red blood cells and 1 part 50% sucrose solution. Human red blood cells had earlier been drawn from people who were not immune to dengue viruses, mixed with an anticoagulant (ethylenediaminetetraacetic acid), and stored in Alsever's solution at 5°C.

The immune serum we used was drawn from a patient approximately 2 months after primary infection with dengue-2 virus. The serum had a hemagglutination inhibition (HAI) titer of 1:80 (Clarke and Casals 1958) and an 80% plaque reduction neutralization titer of 1:80 for dengue-2 virus (Russel et al. 1967); it did not react by neutralization with any other dengue serotypes.

Aedes aegypti were exposed orally by allowing them to feed from hanging drops of the virus–blood suspension, as described by Gubler and Rosen (1976) and Miller et al. (1982). Suspensions were warmed for 4 min in a 37°C water bath before being presented to mosquitoes as drops on the nylon mesh covering the top of the mosquito cage. Only mosquitoes engorged to stage IV or greater on the Pilitt/Jones scale (Pilitt and Jones 1972) were retained for further study. An aliquot of the virus–blood suspension was stored at −70°C for virus titration.

We determined the titer of virus in virus–blood suspensions by inoculating 10-fold dilutions into groups of at least 5 *Ae. aegypti* (Rexville strain). Each mosquito was inoculated with 19 µl of the suspension. After 10 days of incubation at 32°C, recipient mosquitoes were assayed by squashing their heads on microscope slides and assaying them with the direct fluores-

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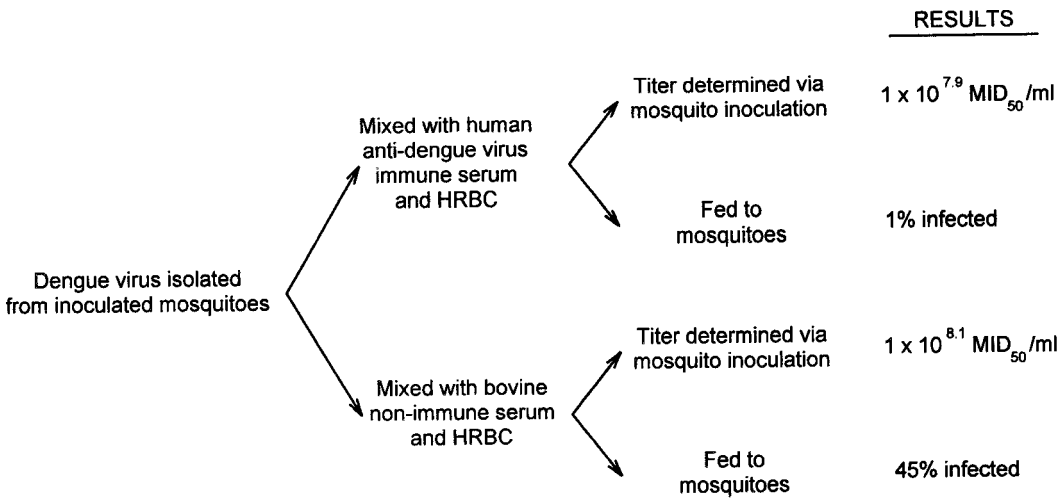

Fig. 1. Summary of the experimental design and results.

cent antibody technique (DFAT) (Kuberski and Rosen 1977). We used the Kärber method to calculate virus titers (Lennette and Schmidt 1979).

Following extrinsic incubation, the infection status of mosquitoes that fed from hanging drops was determined by assaying their squashed heads for viral antigen using the DFAT (Kuberski and Rosen 1977). A paired *t*-test was used on arcsine-transformed data to evaluate the data (Sokal and Rohlf 1987).

Results from our experiment indicate that dengue virus mixed with human immune sera is infectious when inoculated into *Ae. aegypti*. We base this conclusion on the conflicting results of the *per os* infection rates and the titers of the virus–blood suspensions prepared with immune serum (Table 1). Virus in the virus–blood suspensions mixed with immune serum was neutralized, because it infected only 1% of the mosquitoes that imbibed it. This was significantly lower ($0.01 < P < 0.025$, df = 2; Table 1) than the *per os* infection rate of the controls (45%). It was also substantially lower than the 30–90% *per os* infection rates we typically observed during 3 years of studying infection of *Ae. aegypti* with dengue-2 virus (Putnam 1993[3]).

However, when we used the mosquito inoculation technique to titrate the virus content of these neutralized suspensions, we found they had the same titer as the suspensions prepared with nonimmune serum ($P > 0.05$, df = 2; Table 1). That is, the virus–immune serum mixtures were infective when inoculated into a mosquito's hemocoel, but were not infective when imbibed and exposed to gut epithelial cells. Based on the *per os* infection rates, we expected that the virus titer of the blood meals prepared with immune serum ($1 \times 10^{7.9}$ MID_{50}/ml) would be substantially lower than the titers of virus–blood suspensions prepared with nonimmune serum ($1 \times 10^{8.2}$ MID_{50}/ml).

This differential infectivity may be a result of virus–antibody dissociation following inoculation into mosquitoes, as proposed by Rosen et al. (1989), and merits further investigation. If dissociation of virus–antibody complexes does occur within the mosquito's hemocoel, the mode of action might provide a way to improve virus assay techniques and could contribute to an improved understanding of the nature of virus–antibody interactions. Alternatively, differential infectivity may have occurred because: 1) of biochemical changes associated with the trauma of inoculation, 2) infection of cells in the hemocoel is different from cells in the midgut, or 3) differences in the serum (human vs. calf) with which the virus was mixed (Theiler and Downs 1973).

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[3] Putnam, J. L. 1993. The influence of multiple host contacts on the acquisition and transmission of dengue-2 virus by *Aedes aegypti*. Ph.D. Dissertation. University of Maryland, College Park, MD.

Table 1. Infection rates of *Aedes aegypti* that imbibed dengue-2 virus–blood suspensions prepared either with human dengue-2 virus immune serum or nonimmune serum (fetal calf serum), and the virus titer of each suspension as determined by the mosquito inoculation technique.

	Dengue-2 virus–blood suspensions mixed with anti-dengue immune serum		Dengue-2 virus–blood suspensions mixed with nonimmune serum	
Replicate	% infected (+/total)	MID_{50}/ml[1]	% Infected (+/total)	MID_{50}/ml[1]
1	0 (0/28)	$10^{7.8}$	45 (15/33)	$10^{7.9}$
2	0 (0/28)	$10^{7.8}$	59 (27/46)	$10^{8.2}$
3	3 (1/30)	$10^{8.2}$	31 (14/45)	$10^{8.4}$
Mean (total)	1[2] (1/86)	$10^{7.9}$ [3]	45 (56/124)	$10^{8.2}$
SE	1.0	$10^{0.1}$	8.1	$10^{0.1}$

[1] 50% mosquito infectious dose/ml.

[2] Significantly less than mean infection rate of mosquitoes imbibing virus-blood suspensions prepared with nonimmune serum (paired *t*-test; $0.01 < P < 0.025$).

[3] Not significantly different from virus titer of virus-blood suspensions prepared with nonimmune serum (paired *t*-test; $P > 0.05$).

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